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Purification and Characterization of a Trypsin Inhibitor from *Plathymenia foliolosa* Seeds

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A novel trypsin inhibitor (PFTI) was isolated from *Plathymenia foliolosa* (Benth.) seeds by gel filtration chromatography on a Sephadex G-100, DEAE-Sepharose, and trypsin-Sepharose columns. By SDS-PAGE, PFTI yielded a single band with a M_r of 19 kDa. PFTI inhibited bovine trypsin and bovine chymotrypsin with equilibrium dissociation constants (K_i) of 4×10^{-8} and 1.4×10^{-6} M, respectively. PFTI retained more than 50% of activity at up to 50 °C for 30 min, but there were 80 and 100% losses of activity at 60 and 70 °C, respectively. DTT affected the activity or stability of PFTI. The N-terminal amino acid sequence of PFTI showed a high degree of homology with various members of the Kunitz family of inhibitors. *Anagasta kuehniella* is found worldwide; this insect attacks stored grains and products of rice, oat, rye, corn, and wheat. The velvet bean caterpillar (*Anticarsia gemmatalis*) is considered the main defoliator pest of soybean in Brazil. *Diatraea saccharalis*, the sugar cane borer, is the major pest of sugar cane crops, and its caterpillar-feeding behavior, inside the stems, hampers control. PFTI showed significant inhibitory activity against trypsin-like proteases present in the larval midguts on *A. kuehniella* and *D. saccharalis* and could suppress the growth of larvae.

KEYWORDS: ***Plathymenia foliolosa*; trypsin inhibitor; N-terminal sequence; Kunitz family; pest protease**

INTRODUCTION

Proteinase inhibitors (PIs) are found in most organisms, mainly in plants, where they are widely distributed among different families and are particularly abundant (1−10% of the total proteins) in storage organs such seeds and tubers (*1, 2*). They are grouped primarily as either serine, cysteine, aspartic, or metallo-proteinase inhibitors (*3*). Of these, the serine PIs are the most studied and have been isolated from various Leguminosae seeds (*4*). Legume seeds contain various PIs, classified as Kunitz-type, Bowman−Birk-type, potato I, potato II, squash, cereal superfamily, thaumatin-like, and Ragi A 1 inhibitors (*1*). The two best characterized families of plant serine PIs inhibitors are the Kunitz-type and Bowman−Birk inhibitors. These families differ from each other in mass, cysteine content, and number of reactive sites (*5*). Kunitz-type inhibitors are proteins of $M_r \sim 20$ kDa, with low cysteine content and a single reactive site, whereas the Bowman−Birk type inhibitors have $M_r \sim 8-10$ kDa as well as a high cysteine content and two reactive sites (*3*).

Plant-derived PIs are of particular interest because they are part of the plant natural defense system that plants have evolved against insect predation (*6*). Serine PIs are effective against *Lepidoptera* (*7−11*), whereas cysteine PIs are effective against some *Coleoptera* (*12, 13*). These proteins work by blocking digestive proteases in the larval gut, thereby limiting the release of amino acids from food proteins. As a consequence, the larvae display retarded development and eventually die (*14*).

Losses due to insect herbivores, estimated at 10−20% for major crops, are a significant factor in limiting food production (*15*). *Anagasta kuehniella* is a polyphagous pest that feeds on a wide variety of stored products such as dried cocoa beans,

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dried grains, nuts, tobacco, coconut, and dried fruits (*16*). *Anticarsia gemmatalis* remains a key pest of soybean in warm climates (*17*). *Diatraea saccharalis*, the sugar cane borer, is the major insect pest of sugar cane in Brazil and other South American countries (*18*). Control of these insects generally requires the use of chemical insecticides that are toxic to humans and domestic animals and harmful to the environment (*19*).

This paper reports the purification, characterization, and inhibitory properties of a trypsin inhibitor from *Plathymenia foliolosa* (Benth.) seeds, a Leguminosae tree (subfamily Mimosoideae) with occurrence from Pernambuco to Rio de Janeiro. The inhibitory activity toward trypsin-like proteinases extracted from different lepidopteran larvae such as *Ana. kuehniella* (Zeller), *D. saccharalis* (Fabr.), and *Ant. gemmatalis* (Hueb.) was also studied, because these insect pests cause great economic loss in crops.

MATERIALS AND METHODS

Materials. *P. foliolosa* seeds were colleted in the city of Campinas, in the State of São Paulo (Brazil). Bovine serum albumin (BSA), *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-benzoyl-L-tyrosyl-*p*-nitroanilide (BTPNA), *N*-*p*-tosyl-lysine chloromethyl ketone (TLCK), and tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma (St. Louis, MO), as were SDS-PAGE molecular weight markers, acrylamide, bis-acrylamide, and other electrophoresis reagents. Chromatography supports were from Pharmacia (Uppsala, Sweden). All other chemicals and reagents used were of analytical grade.

Purification of *P. foliolosa* Trypsin Inhibitor (PFTI). *P. foliolosa* seeds (200 gr) free from integument (which was removed manually using a stylus) and defatted with hexane absolute were ground in a coffee mill. A crude inhibitor preparation was obtained by extraction of this meal with 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl (1:20, w/v) for 24 h at 4 °C with subsequent centrifugation at 5000*g* for 30 min. The supernatant was dialyzed against distilled water for 24 h at 4 °C and lyophilized. This material (300 mg) was dissolved in 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl and applied to a Sephadex G-100 column (2 × 50 cm) equilibrated with the same buffer. Fractions (3 mL) from the protein peak were collected at a flow rate of 40 mL/h. Proteinase inhibitory activities were checked, and the fraction with maximum inhibitory activity (50 mg) was applied to an ion-exchange DEAE-Sepharose column (2 × 20 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0. Fractions (3 mL) were collected using a step gradient (0−1 M) of NaCl at a flow rate of 0.6 mL/min. The peak (DEAE II) obtained was pooled, dialyzed, lyophilized, and applied (30 mg) to a trypsin−Sepharose column (2 × 10 cm) equilibrated with 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl. The retained proteins were eluted with 100 mM HCl solution at flow rate of 30 mL/h and dialyzed against distilled water (*20*). The cut of dialyses was 10 kDa. The antitryptic peak (PFTI) was pooled and concentrated for further analyses. Proteins were detected by monitoring the absorbance at 280 nm.

Assay of Inhibitory Activity. Trypsin inhibitory activity was determined by measuring the residual hydrolytic activity of bovine trypsin TPCK-treated and chymotrypsin TLCK-treated samples toward the substrates BAPNA and BTPNA, respectively. Both of the proteinases were preincubated with PFTI (1 mg/mL) for 10 min at 37 °C, in 1 mL of Tris-HCl buffer (50 mM), pH 8.0, prior to the reaction (*21*). To start the reaction, BAPNA and BTPNA solution were then added to the mixture. After 10 min, the reaction was stopped by the addition of 30% (v/v) acetic acid. One inhibitor unit was defined as the amount of inhibitor that inhibited 1 unit of enzyme activity.

Protein Quantification. Protein content was determined by Coomassie Blue staining according to the procedure of Bradford (*22*) as well as from the absorbance at 595 nm. BSA (1 mg/mL) was used as a protein standard.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) in the absence and presence of dithiothreitol (DTT, 100 nM) was done as described by Laemmli (*23*). The proteins used as molecular weight standards were phosphorylase (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). The proteins were detected by staining with 0.1% Coomassie Brilliant Blue R-250.

Stoichiometry of Trypsin and Chymotrypsin Inhibition. The dissociation constant (K_i) and the inhibitor concentration were determined for bovine trypsin and chymotrypsin by preincubating the respective enzymes with increasing concentrations of purified inhibitor (1 mg/mL) in 100 mM Tris-HCl (pH 8.0), 37 °C, followed by measurement of the residual activity using appropriate substrates (BAPNA and BTPNA, respectively). Apparent values of K_i were determined by adjusting the experimental points to the equation for slow tight binding (*24*), using a nonlinear regression with the help of the Enzfitter program.

Formation of PFTI−Trypsin Complexes. The PFTI−trypsin complexes were gel filtered (0.3 mL/min) on a Sephadex G-100 column equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl. Trypsin and inhibitor (1:1 molar ratio), trypsin−inhibitor complexes, and trypsin in a molar excess of the enzyme were preincubated for 10 min at 37 °C in 100 mM Tris-HCl buffer, pH 8.0, with 0.02% $CaCl_2$. As a control, isolated proteins were gel filtered as described above. The elution profiles were monitored on the basis of the absorbance at 280 nm, and the inhibitory activity was followed using BAPNA as substrate. The column was calibrated with albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsinogen (24 kDa), and cytochrome *c* (12 kDa).

Temperature and pH Stability. The inhibitor solution (1 mg/mL of 50 mM Tris-HCl buffer, pH 8.0), was heated for 30 min in a water bath at various temperatures (37−100 °C) and then cooled to 0 °C before testing for residual inhibitory activity. To measure the pH stability, a solution of inhibitor (1 mg/mL) was diluted with an equal volume of various buffers (100 mM): sodium citrate (pH 2−4), sodium acetate (pH 4.5−5.5), sodium phosphate (pH 6−7), Tris-HCl (pH 7.5−8.5), and sodium bicarbonate (pH 9−10). After incubation in each buffer for 1 h at 37 °C, the pH was adjusted to pH 8.0 and the inhibitory activity on trypsin was assayed as described below.

Effect of DTT. The inhibitor (1 mg/mL) was incubated with the reducing agent DTT at final concentrations of 1, 10, and 100 mM for 15−120 min at 37 °C. The reaction was terminated by adding the amount of iodoacetamide twice in relation to each DTT concentration, and the residual inhibitory activity on trypsin was then determined. After the treatments above, the residual inhibitory activity on trypsin was measured using BAPNA as substrate. Aliquots (50 μL of trypsin inhibitor solution) were mixed with a stock solution of bovine trypsin (50 μL, 0.33 mg/mL in 2.5 mM HCl) in 50 mM Tris-HCl buffer, pH 8.0. The mixture was incubated at 37 °C for 10 min followed by the addition of 1 mL of BAPNA (100 mM) to give a final volume of 1.5 mL. After 20 min of incubation, the reaction was stopped by adding 200 μL of 30% (v/v) acetic acid. Substrate hydrolysis was followed by the increase in absorbance at 410 nm. All experiments were done in triplicate, and the results are the mean of three assays.

N-Terminal Amino Acid Sequence. The N-terminal amino acid sequence of PFTI was determined by direct sequencing using automated Edman degradation in a PROCISE amino acid sequencer (Applied Biosystems). The phenylthiohydantoine (PTH) amino acids were identified in a model 140C microgradient PTH amino acid analyzer (Waters system), on the basis of their retention times. The sequence was submitted to automatic alignment, which was performed by using NBCI-Blast search system.

Insects. The eggs and larvae of *Ana. kuehniella*, *D. saccharalis*, and *Ant. gemmatalis* were originally supplied by Dr. J. R. P. Parra (Laboratório de Biologia dos Insetos, Escola Superior de Agronomia "Luiz de Queiroz" Universidade São Paulo, Piracicaba, SP, Brazil). These insects were housed at 28 ± 1 °C, in a relative humidity of 65−75% (16 h photophase) and were maintained in the laboratory until the fourth instar. *Ana. kuehniella* was fed a standard artificial diet prepared by mixing whole wheat flour, whole wheat husks, whole wheat, and yeast (8:2:1.9:0.1, w/w). The composition of the artificial diet for *D. saccharalis* and *Ant. gemmatalis* was similar to that followed by Telang et al. (*25*) and chiefly composed of (for 0.5 L of distilled water) 55.0 g of wheat germ, 8.5 g of agar−agar, 55.0 g of kidney

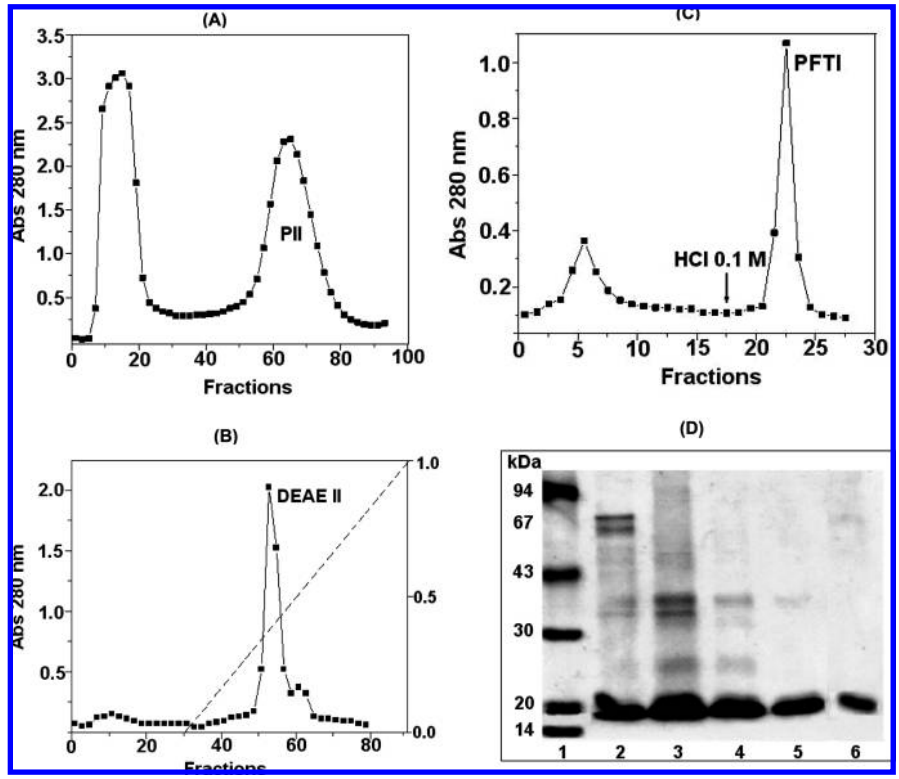

Figure 1. (**A**) Gel filtration (Sephadex G-100) of total extract. PII contained the trypsin-inhibiting activity and was subjected to ion-exchange chromatography (**B**) on a DEAE-Sepharose column (2 × 20 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The fraction eluting after the saline gradient (DEAE II) was applied to a trypsin−Sepharose affinity column (2 × 10 cm) (**C**) equilibrated with 100 mM phosphate buffer, pH 7.6, containing 100 mM NaCl. (**D**) SDS-PAGE showing all fractions obtained during purification: lane 1, molecular weight marker; lane 2, total extract; lane 3, Sephadex G-100; lane 4, DEAE-Sepharose; lane 5, trypsin−Sepharose; lane 6, fraction PFTI reduced with 0.1 M DTT.

bean flour, 20.0 g of dried yeast powder, 2.5 g of casein, 3.5 g of ascorbic acid, 0.35 g of methyl-*p*-hydroxybenzoate, 0.75 g of sorbic acid, 80 mg of streptomycin sulfate, 0.25 g of cholesterol, 0.7 mL of multivitamin drops, and vitamin B complex (0.2 g).

Midgut Preparation. Proteinases were obtained from the midguts of fourth-instar larvae, according to a protocol by Macedo et al. (*26*). The larvae were cold-immobilized and dissected, and the midguts were surgically removed and placed in iso-osmotic saline (150 mM NaCl solution). The guts and contents were stored at −20 °C until use. Guts from larvae of *Ana. kuehniella*, *D. saccharalis*, and *Ant. gemmatalis* were subsequently homogenized in 150 mM NaCl and centrifuged at 6000*g* for 5 min, and supernatants were cooled and kept on ice for enzymatic assays. The larvae of fourth-instar were used for its present larger enzymatic activity during the larval development of the insect.

Effects of PFTI on Endogenous Proteolytic Activity. The effect of PFTI on the proteolytic activity of whole midgut extracts was measured by using BAPNA (1 mM) as substrate. The assays were run at pH 8.0 in 100 mM Tris-HCl. PFTI (4−36 μg) was incubated with the midgut extracts (50 μg of protein) at 37 °C for 15 min before addition of the substrate. The reaction was stopped by adding 30% (v/v) acid acetic. All incubations were done in triplicate, and appropriate controls were included. The resulting absorbance was read at 410 nm.

Statistical Analysis. All data were examined using one-way analysis of variance (ANOVA) (General Linear Models on GLM procedure). The Student−Neuman−Keul's test was used to identify the means that differed if the ANOVA test indicated significance. A p value of <0.05 was considered to be significant.

RESULTS

Purification and Properties of PFTI. PFTI was purified by extraction in 100 mM phosphate buffer (pH 7.6), gel filtration on Sephadex G-100, ion-exchange chromatography on DEAE-Sepharose, and affinity chromatography on trypsin−Sepharose. Only one peak from gel filtration on G-100 (**Figure 1A**) and DEAE-Sepharose (**Figure 1B**) showed antitryptic activity. The peak eluting after the saline gradient was rechromatographed on an affinity column (**Figure 1C**) of enzymatically inactive trypsin−Sepharose and yielded only one peak (PFTI) with antitryptic activity. Affinity chromatography proved to be a very convenient step to isolate this inhibitor.

Chemical Characterization. SDS-PAGE in the absence and presence of DTT (0.1 M) (**Figure 1D**) showed that PFTI consisted of a single polypeptide chain with a molecular mass of approximately 19 kDa, which was also confirmed by gel filtration chromatography on Sephadex G-100. The K_i of 4×10^{-8} M obtained from the inhibitor with bovine trypsin TPCK (**Figure 2A**) and the $K_i = 1.4 \times 10^{-6}$ M obtained from the inhibitor with bovine chymotrypsin TLCK (**Figure 2B**) were calculated using the equation for slow-tight binding inhibition (*24*) and nonlinear regression with the help of the Enzfitter program.

Stoichiometric studies, based on gel filtration, showed that PFTI, incubated with trypsin at a 1:1 molar ratio, produced a complex with a molecular mass of approximately 42 kDa (**Figure 3**), which corresponded to combined masses of the PFTI and of the enzyme separately; no peak was found with inhibitory activity, suggesting that PFTI is capable of inhibiting only one enzyme molecule at a time. The database search, using the N-terminal sequence of PFTI, revealed homology with Kunitz-type trypsin inhibitors, mainly with the *Acacia confusa* inhibitor and *Prosopsis juliflora* inhibitor (**Figure 4**).

Stability of Inhibitory Activity. The study of the effect of temperature on the inhibitor showed that the inhibitor retained >50% of activity at up to 50 °C for 30 min, but there were 80 and 100% losses of activity at 60 and 70 °C, respectively

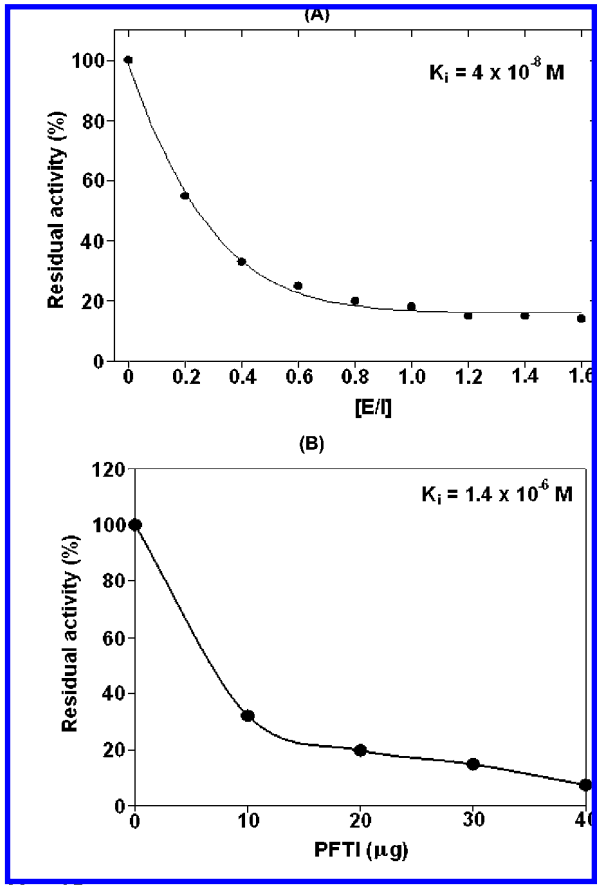

Figure 2. Titration curve of bovine trypsin TPCK (**A**) and bovine chymotrypsin TLCK (**B**) inhibition by PFTI. Increasing concentrations of inhibitor were added to a fixed concentration of enzyme. Residual enzyme activity was determined by using BAPNA and BTPNA as substrate, respectively. Each point is the mean of three assays.

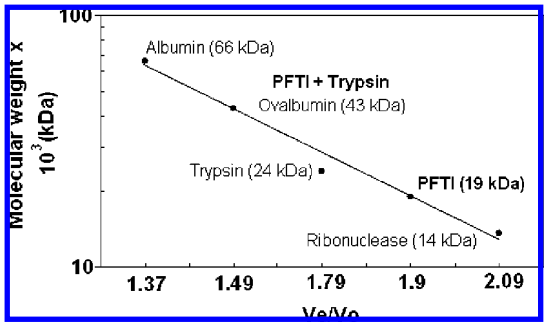

Figure 3. Determination of the molecular weight of the complex trypsin−PFTI in Sephadex G-100 gel filtration. Samples were incubated for 10 min at 37 °C, equilibrated with 0.1 M phosphate buffer, pH 7.6, 0.1 M NaCl.

(**Figure 5A**). Preincubation of the inhibitor in the pH range of 2.0−10.0 for 60 min affected trypsin inhibition. Results demonstrate that the inhibitor loses its stability in extreme conditions of pH, remaining active at pH 6.0−8.0 (**Figure 5B**). **Figure 6** shows that there was a loss of inhibitory activity of about 50% following exposure to different concentrations of DTT.

Effects of PFTI on Endogenous Proteolytic Activity. Figure **7** shows the in vitro specificity of PFTI against trypsin-like activity from larval midguts of insect pests of economic importance belonging to the order Lepidoptera. The larval digestive enzymes extracted from the *Ana. kuehniella* and *D. saccharalis* were clearly inhibited by PFTI, whereas the inhibitory activity of PFTI inhibited the tryptic activity of *Ant. gemmatallis* only moderately.

DISCUSSION

Serine proteinase inhibitors and their binding to cognate proteinases have been extremely well characterized and, in particular, the Kunitz family trypsin inhibitors from a variety of plant sources have received special attention over the years (*27*). Several such Kunitz PIs have been shown to impart protection to host plants by having detrimental effects on the growth and development of their insect pests (*7, 28*−*30*).

The present study describes the purification and biochemical properties of a new trypsin inhibitor from *P. foliolosa* seeds, as well as its effects on digestive proteinases from insect pests. The PFTI is a protein of approximately 19 kDa molecular mass as observed by SDS-PAGE analysis with only one polypeptide chain in the presence of reducing agent, similar to trypsin inhibitors of the Kunitz family, which generally have molecular masses of 18−24 kDa composed by one or two polypeptide chain (*16, 31*−*33*). The results describing PFTI as a protein composed of single polypeptide chain agree with those described for most Kunitz-type inhibitors of the subfamily Mimosoideae such as those purified from *Inga laurina* and *Dimorphandra mollis* seeds (*21, 31, 34*).

The specificity of the Kunitz inhibitors varies among the subfamilies of the Fabaceae family. Some Kunitz-type inhibitors isolated from the most primitive species of the Caesalpinioideae subfamily have only activity for trypsin (*16, 35*− *37*), and others from the Papilionoideae or Faboideae subfamily contain chymotrypsin inhibitors (*1, 38, 39*), but Kunitz-type inhibitors are also found (*29, 3040*). The inhibitory activity of PFTI was assessed by using different enzymes. PFTI inhibited bovine trypsin TPCK ($K_i = 4 \times 10^{-8}$ M), but affected bovine chymotrypsin TLCK only weakly ($K_i = 1.4 \times 10^{-6}$ M). This K_i value for trypsin indicates a high affinity between the enzyme and inhibitor, as is also shown for other plant trypsin inhibitors (*34, 41*).

The reactive site of a protease inhibitor is defined as that part of the molecule entering into direct contact with the active center of the enzyme to form an enzyme−inhibitor complex (*42*). When PFTI was incubated with trypsin at a 1:1 molar ratio, a complex with a molecular mass of approximately 42 kDa was observed by gel filtration on Sephadex G-100. This experiment showed that a ternary complex was not formed and confirmed the presence of a single reactive site for trypsin. The stoichiometry ratio of 1:1 and the molecular mass agreed with those for other Kunitz inhibitors (*12143*−*45*).

The intramolecular disulfide bridges are presumably responsible for the functional stability of Kunitz-type inhibitors in the presence of various physical and chemical denaturants (*46*) such as temperature, pH, and reducing agents. Thermal inactivation of PFTI at different temperatures resulted in progressive loss of trypsin-inhibiting activity at temperatures >40 °C, and a greater decrease in activity was observed when heated to 60 and 70 °C. Displaying wide pH amplitude, PFTI is not stable under conditions ranging from highly acidic to high alkaline. In fact, the inhibitor maintained ∼80% of its inhibitory activity through a pH gradient of 6.0−8.0. DTT affected the activity or stability of PFTI, similar to the findings of Ramasarma et al. (*47*), who studied a Bowman−Birk inhibitor from *Dolichos biflorus*. Macedo et al. (*16*) observed that 100 mM DTT reduced the inhibitory activity of PDTI, a Kunitz-type trypsin inhibitor from *Peltophorum dubium*. The reduction of the inhibitory

	Initial Position	Sequence/homology	Similarity (%)
PFTI	1	F E L K D M E G D I L E N G G L Y Y I V P A S	--------
ACACO	2	E L L D A D G D I L R N G G A Y Y I L P A	88%
PROJU	2	E L L D V D G E I L R N G G S Y Y I L P A	83%
ADEPA	2	E L L D V D G N F L R N G G S Y Y I V P A	77%
PDTI	1	D F V L D A E G K F L L N G G I Y Y I L	55%
PSOTE	2	E L V D V E G K T V R N G G T Y Y L V P	66%
ERYVA	1	E L V D V E G E D V V N G G T Y Y M L P	61%
SBTI	1	D F V L D N E G N P L S N G G T Y Y I L	55%
DMTI-I	2	Q V F D T E G N G I R N G G T Y Y I L P D R	55%

Figure 4. Comparison of the N-terminal sequence of the PFTI aligned with different regions of known Kunitz trypsin inhibitors. ACACO, *Acacia confusa* trypsin inhibitor (*48*); PROJU, *Prosopsis juliflora* trypsin inhibitor (*49*); ADEPA, *Adenanthera pavonina* trypsin inhibitor (*50*); PDTI, *Peltophorum dubium* trypsin inhibitor (*16*); PSOTE, *Psophocarpus tetragonolobus* trypsin inhibitor (*51*); ERYVA, *Erythrina variegata* trypsin inhibitor (*52*); SBTI, soybean (*Glycine max*) trypsin inhibitor (*53*); DMTI-I, *Dimorphandra mollis* trypsin inhibitor (*34*). Identical residues are in shaded blocks.

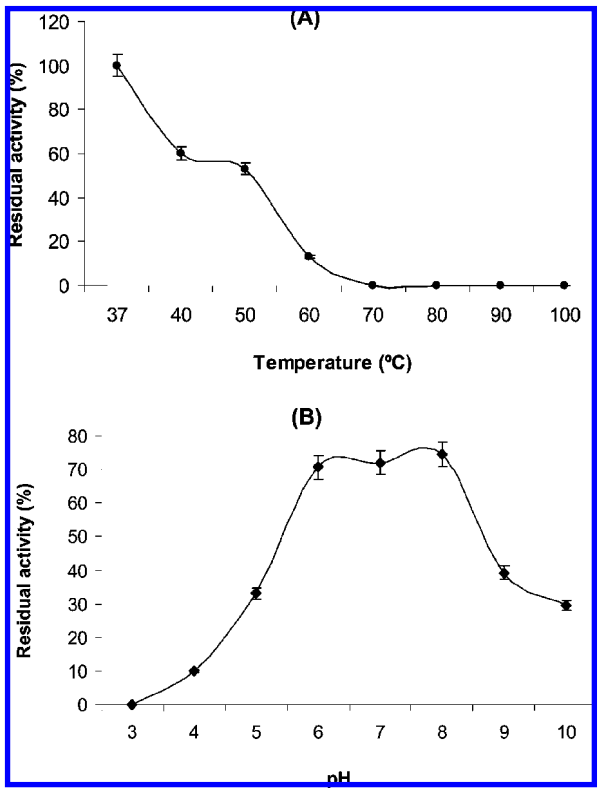

Figure 5. Stability of PFTI: (**A**) temperature stability of the inhibitory activity of PFTI after incubation for 30 min at the indicated temperature; (**B**) pH stability of PFTI. The activity was assayed using BAPNA in 0.05 M Tris-HCl, pH 8.0, after incubation at the indicated pH for 30 min at 37 °C. The points are the means ± SE of three assays.

activity probably happens for the break of the intramolecular disulfide bridges.

The partial amino-terminal sequence of the first 22 amino acids of PFTI revealed many conserved residues and a high degree of homology with Kunitz type inhibitors (KTIs) of Mimosoideae. The amino-terminal sequences of KTIs are conserved. Comparison of the amino-terminal region of PFTI with that other inhibitors showed that there were many conserved residues with 55−88% identity between KTIs. The amino-terminal of PFTI contained 18 residues of consensus sequence of invariable residue (-EL-D-EGDILENGG-YYIV-PA), which are also present in all KTIs (*16, 34, 48*−*53*).

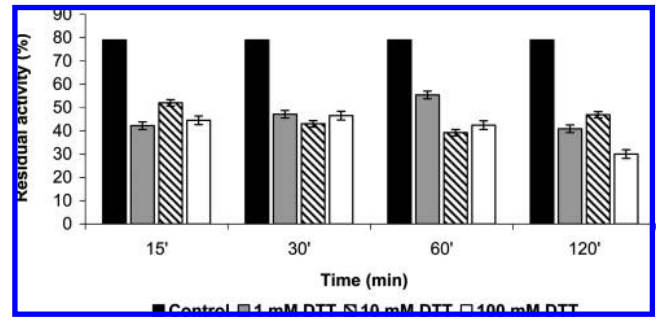

Figure 6. Effect of DTT on the stability of PFTI. The inhibitor was treated with different final concentrations (1, 10, and 100 mM) of DTT for 15−120 min, at 37 °C. The reaction was interrupted with twice the amount of iodoacetamide for each DTT concentration, and the residual trypsin inhibitory activity was measured using BAPNA as substrate. The columns are the means ± SE of three assays.

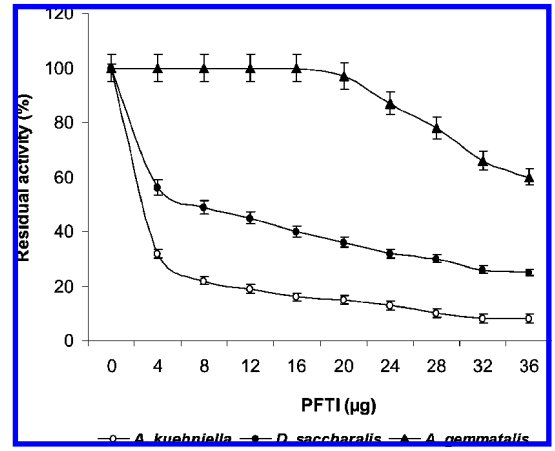

Figure 7. Inhibition by PFTI of the midgut proteolytic activity (assayed with BAPNA) of fourth-instar larvae. The points are the means ± SE of three separates tests with midgut extracts.

The phytophagous larvae of most lepidopteran species analyzed so far have alkaline midgut fluids, with serine proteases and exopeptidases providing most of the midgut proteolytic activity (*54, 55*). PIs have been considered as natural control agents against herbivorous insects because they reduce proteolytic enzyme activity in vitro and affect larval development in a number of lepidopteran species. However, not all insect species are susceptible to PIs, even

though they secrete digestive enzymes that can be inhibited in vitro by theses inhibitors (*56, 57*).

PFTI was assayed in vitro against proteases from three species of insect pests—*Ana. kuehniella*, *D. saccharalis*, and *Ant. gemmatallis*, all belonging to the order Lepidoptera, in order to assess the general hypothesis that serine PIs can protect plants against herbivorous insects (*7, 58*−*60*). PFTI was more active against *Ana. kuehniella* and *D. saccharalis* and inhibited the tryptic activity of *Ant. gemmatalis* only moderately. A number of Kunitz-related inhibitors from plants were evaluated in vitro for their potential of decreasing the activity of Lepidopteran/Coleopteran/Dipteran larvae digestive enzymes (*61*). The digestive enzymes from larvae of *Zabrotes subfasciatus* and *Ceratitis capitata* were inhibited by PdKI, the first Kunitz trypsin inhibitor purified from *Pithecelobium dumosum* seeds (*62*). Soybean Kunitz trypsin inhibitor (SKTI) showed a potent in vitro inhibitory activity against *Anthonomus grandis* gut proteinases (*63*). Inhibitors of proteinases have been used successfully for protection of engineered resistance toward insect pests (*7, 64, 65*), but this effect is lost when insect pests develop escape mechanisms.

When expressed in transgenic plants at levels from tenths of a percent to several percents of total soluble protein, PIs have been shown to confer some protection to plants against insect pests, mainly Lepidoptera (*60*). Falco and Silva-Filho (*6*) used SKTI and soybean Bowman−Birk inhibitor (SBBI) to illustrate that the genes encoding insect antinutritional proteins can be both transferred to and adequately expressed in transgenic sugar cane. This was the first report of the transformation of sugar cane with proteinase inhibitor genes, which has resulted in conferring some level of resistance to insect damage.

Our studies on the effects of PFTI on insect gut proteinases reflect that PFTI is a powerful antifeedant of herbivorous insects. An in-depth analysis of the diverse biological activities, such as toxicity, sequestration, and unpalatability, of PFTI on the growth and development of different crop pests needs to be carried out to confirm the biotechnological potential of PFTI as an agent against phytophagous insects.

ABBREVIATIONS USED

BSA, bovine serum albumin; DEAE, diethylaminoethyl; DTT, dithiothreitol; KTIs, Kunitz type inhibitors; BAPNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; BTPNA, *N*-benzoyl-L-tyrosyl-*p*-nitroanilide; TLCK, *N*-*p*-tosyl-lysine chloromethyl ketone; PdKI, *Pithecelobium dumosum* Kunitz trypsin inhibitor; PFTI, *Plathymenia foliolosa* trypsin inhibitor; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SBBI, soybean Bowman−Birk inhibitor; SKTI, soybean Kunitz trypsin inhibitor; TPCK, tosyl-L-phenylalanine chloromethyl ketone.

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